Response and Resistance to NF-κB Inhibitors in Mouse Models of Lung Adenocarcinoma

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Lung adenocarcinoma is a leading cause of cancer death worldwide. We recently showed that genetic inhibition of the NF-κB pathway affects both the initiation and the maintenance of lung cancer, identifying this pathway as a promising therapeutic target. In this investigation, we tested the efficacy of small-molecule NF-κB inhibitors in mouse models of lung cancer. In murine lung adenocarcinoma cell lines with high NF-κB activity, the proteasome inhibitor bortezomib efficiently reduced nuclear p65, repressed NF-κB target genes, and rapidly induced apoptosis. Bortezomib also induced lung tumor regression and prolonged survival in tumor-bearing KrasL5-G12D/wt, p53floxflox mice but not in KrasL5-G12D/wt mice. After repeated treatment, initially sensitive lung tumors became resistant to bortezomib. A second NF-κB inhibitor, Bay-117082, showed similar therapeutic efficacy and acquired resistance in mice. Our results using preclinical mouse models support the NF-κB pathway as a potential therapeutic target for a defined subset of lung adenocarcinoma.

SIGNIFICANCE: Using small-molecule compounds that inhibit NF-κB activity, we provide evidence that NF-κB inhibition has therapeutic efficacy in the treatment of lung cancer. Our results also illustrate the value of mouse models in validating new drug targets in vivo and indicate that acquired chemoresistance may later influence bortezomib treatment in lung cancer. Cancer Discovery; 1(3): 236–47. ©2011 AACR.

INTRODUCTION

Lung cancer is the leading cause of cancer death worldwide, with 1.3 million people projected to die of this disease in the next year (1). Non–small-cell lung cancer (NSCLC) represents 85% of lung cancer cases. Lung adenocarcinoma, a histologic class of NSCLC, is associated with recurrent mutations in several well-defined oncogenes and tumor suppressor genes. Oncogenic KRAS mutations occur in 25% of lung adenocarcinomas, and inactivating mutations in the tumor suppressor gene p53 (TP53) are found in 50% of cases (1).

The 5-year survival rate of individuals diagnosed with lung cancer in the United States is poor, at only ~15%, and the prognosis is even worse for individuals who have a diagnosis of advanced disease (2). Although much effort has been devoted to developing targeted therapies for lung cancer, few have proven effective thus far (3). Recent successful targeted therapies include the epidermal growth factor receptor (EGFR) inhibitor gefitinib/erlotinib for patients with EGFR mutation (4) and anaplastic lymphoma kinase (ALK) inhibitors for patients with EML4-ALK translocations (5). Yet, to date, no targeted therapies have been used effectively against KRAS mutant lung cancer.

The NF-κB pathway is an emerging cancer drug target (6, 7). The mammalian NF-κB transcription factor family comprises 5 subunits: RELA (p65), RELB, REL (cRel), NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100), which form homodimers or heterodimers (8). Two major NF-κB pathways, canonical and alternative, have been well characterized (9). In the canonical pathway, NF-κB (usually composed of a p65-p50 heterodimer) is inhibited through sequestration in the cytoplasm by the inhibitor of κB (IκB) under nonstimulated conditions. IκB is a target of several upstream signaling cascades that activate an IκB kinase (IKK) complex composed of at least 2 kinases, IKKα and IKKβ, and of 1 regulatory subunit, NF-κB essential modulator (NEMO; also called IKKγ). Both IKKα and IKKβ can directly phosphorylate IκB, resulting in its ubiquitination and degradation by the 26S proteasome (7). Once released from IκB, NF-κB becomes active through nuclear translocation and DNA binding. In the alternative pathway, IKKα, activated by NF-κB–inducing kinase, phosphorylates p100, resulting in limited degradation of p100 into p52 by the proteasome, followed by nuclear translocation of the RELB-p52 heterodimer (6).

The NF-κB pathway shows promise for cancer therapy because NF-κB transcription factors are crucial regulators of mechanisms associated with tumorigenesis, and their multifaceted functions are achieved through regulation of NF-κB target genes (6, 10). NF-κB target genes are associated with numerous hallmarks of cancer (11), including inflammation (TNF, IL6, IL1, ICAM1, and MCP1), proliferation (MYC, CYCLIND1, CYCLINE2, and CDK2), survival (BCL2, BCLxL, cIAP1/2, XIAP, and SURVIVIN), tumor progression (MMP2/9 and COX2), angiogenesis (HIF1α and VEGF), and cell death (FAS and FASL). Because NF-κB regulates a panel of key

ABSTRACT

Lung adenocarcinoma is a leading cause of cancer death worldwide. We recently showed that genetic inhibition of the NF-κB pathway affects both the initiation and the maintenance of lung cancer, identifying this pathway as a promising therapeutic target. In this investigation, we tested the efficacy of small-molecule NF-κB inhibitors in mouse models of lung cancer. In murine lung adenocarcinoma cell lines with high NF-κB activity, the proteasome inhibitor bortezomib efficiently reduced nuclear p65, repressed NF-κB target genes, and rapidly induced apoptosis. Bortezomib also induced lung tumor regression and prolonged survival in tumor-bearing KrasL5-G12D/wt, p53floxflox mice but not in KrasL5-G12D/wt mice. After repeated treatment, initially sensitive lung tumors became resistant to bortezomib. A second NF-κB inhibitor, Bay-117082, showed similar therapeutic efficacy and acquired resistance in mice. Our results using preclinical mouse models support the NF-κB pathway as a potential therapeutic target for a defined subset of lung adenocarcinoma.

SIGNIFICANCE: Using small-molecule compounds that inhibit NF-κB activity, we provide evidence that NF-κB inhibition has therapeutic efficacy in the treatment of lung cancer. Our results also illustrate the value of mouse models in validating new drug targets in vivo and indicate that acquired chemoresistance may later influence bortezomib treatment in lung cancer. Cancer Discovery; 1(3): 236–47. ©2011 AACR.
oncogenes (e.g., MYC) and prosurvival genes (e.g., BCL2), this pathway has been implicated in tumor initiation, progression, and resistance to chemotherapy (12). Aberrant NF-κB pathway activity has been frequently observed in human cancer through cancer genomic studies. For example, mutations in the NF-κB pathway are detected in >20% of multiple myelomas (13) and are potentially involved in lung cancer (14). In diffuse large B-cell lymphomas (DLBCL), NF-κB mutations are found in >50% of the activated B-cell-like (ABC) subtype but rarely in the germinal center B-cell-like (GCB) subtype (15). Consistent with these observations, IKK inhibitors showed cytotoxicity selectively in ABC-DLBCL cell lines, but not in GCB-DLBCL cells (16).

Although small-molecule compound inhibitors of NF-κB have been proposed as rational single-agent therapies for cancers with aberrant NF-κB activity, most classic NF-κB inhibitors are poorly selective and have known off-target effects (6, 17). Because proteasome-mediated degradation of IkB is a required step in NF-κB signaling, the proteasome inhibitor bortezomib (Velcade/PS-341) has been proposed as a general inhibitor of NF-κB (6, 7). Bortezomib is an FDA-approved first-line treatment for advanced multiple myeloma, a disease with frequent NF-κB pathway activation (18-21). In multiple myeloma studies, patients with high levels of NF-κB are more sensitive to bortezomib (22), suggesting that although proteasome inhibition may affect other signaling pathways, NF-κB is an essential target of this drug (6). A second NF-κB inhibitor, Bay-117082, was identified as a compound inhibiting cytokine-induced IkB phosphorylation (23). Like bortezomib, Bay-117082 has been shown to suppress NF-κB signaling in vitro and in vivo (23, 24). This compound, although not clinically approved, has been studied in mouse lymphoma models (24).

Mouse models of human cancer are powerful tools to study tumor biology, genetics, and therapies. Previously, mouse models of Eμ-Myc B-cell lymphoma were successfully used to examine the chemotherapy response (25). Similar studies in mouse models of lung cancer have led to new insights into the activity of PI3K inhibitors (26) and cisplatin in vitro (2). Our laboratory has developed an autotransplant mouse model of human lung cancer, in which lung tumors are initiated upon Cre recombinase-mediated activation of a KrasG12D allele. In this case, KrasG12D activation alone (KrasG12D/WT, K model) generates low-grade adenocarcinomas (27). When combined with the concomitant loss of both p53 alleles (KrasG12D/WT, p53flox/flox, KP model), the mice develop lung tumors with a shorter latency and advanced histopathology (28, 29). These models are thus suitable for evaluating novel targeted small-molecule compounds in a physiological setting.

We previously showed that activation of Kras and loss of p53 selectively activate NF-κB and that genetic inhibition of the NF-κB pathway in tumor epithelial cells resulted in significantly delayed lung tumor progression (30). Similar genetic studies have revealed that p65/RelA is required for KrasG12D-induced lung tumorigenesis (31) and that Gprc5a loss enhances NF-κB activation in lung epithelial cells and promotes tumorigenesis (32). These results indicate a critical function for NF-κB signaling in lung tumor development and suggest NF-κB inhibitory drugs as potential targeted therapies for lung cancers with mutations in Kras and p53 or with activation of the NF-κB pathway. In this article, we describe the short-term and long-term effects of 2 general NF-κB inhibitors, bortezomib and Bay-117082, in the K and KP models of lung adenocarcinoma. The results indicate that small-molecule inhibition of this pathway can cause tumor regression but that long-term treatment is associated with acquired resistance.

RESULTS

Bortezomib Inhibits NF-κB Signaling and Induces Apoptosis in Lung Adenocarcinoma Cells

Bortezomib has been shown to inhibit NF-κB by suppressing proteasome-mediated IkB degradation (19, 33). We thus hypothesized that bortezomib would inhibit NF-κB signaling in lung adenocarcinoma cells. We have generated a panel of genetically defined mouse lung adenocarcinoma cell lines (hereafter termed “KP”) from tumors carrying a Cre-activatable KrasG12D allele (KrasG12D/WT, LSL denotes Lox-stop-Lox) and a conditional loss-of-function p53 allele (p53flox/flox). As shown in Fig. 1A, bortezomib-treated cells, compared with vehicle control-treated cells, showed reduced nuclear accumulation of the NF-κB transcription factor subunit p65 (also known as RelA). Because nuclear p65 is required for NF-κB activity, these data suggest that bortezomib is able to inhibit the NF-κB pathway in mouse lung adenocarcinoma cells.

To explore the molecular and cellular effects of NF-κB inhibition in KP cells, we examined the expression levels of known NF-κB target genes by real-time PCR after a time course of bortezomib treatment (Fig. 1B). Of note, NF-κB-regulated antiapoptosis genes, such as Bcl2, Bcl-xl, Birc5 (cIAP1), and Birc3 (Survivin), were consistently downregulated at all time points tested, demonstrating the efficiency of NF-κB inhibition. Furthermore, proliferation-related NF-κB target genes, including c-Myc and Cyclin D1, also reduced expression following bortezomib treatment (Fig. 1B). Contrary to our expectations, the expression of 3 NF-κB targets that regulate inflammation, Il6, Tnf, and Mmp3, was not reduced after treatment, and the Il6 mRNA level was actually increased in cells treated with bortezomib. Induction of the inflammatory genes may be due to secondary proinflammatory effects of NF-κB inhibition in these cells. Future experiments will examine the importance of bortezomib’s proinflammatory effects in tumor cells.

As the NF-κB pathway is known to inhibit apoptosis through its regulation of antiapoptotic genes, we next addressed the cytotoxicity of bortezomib in vitro (6). Consistent with decreased levels of Bcl2 and other antiapoptotic genes in bortezomib-treated cells (Fig. 1B), we observed increased cleaved caspase 3 (CC3) in KP, as well as in cells harboring the KrasG12D mutation and a point mutation (R172H) in p53 (KPWT, T.G. Oliver and T. Jacks, unpublished data; ref. 34; Fig. 1C). We also performed Trypan blue counting and confirmed that bortezomib treatment caused cell death in a dose-dependent...
manner in KP and KP\textsuperscript{M} cells. Of interest, LKR13 cells, which express mutant Kras but retain wild-type p53 expression and 3TZ fibroblasts (30), did not show substantial cell death under the assayed conditions.

We also tested bortezomib in 2 human NSCLC cell lines that contain mutations in \textit{KRAS} and loss of function in \textit{p53} (Supplementary Fig. S1). Consistent with the mouse data, the NF-\kappa B target genes \textit{MYC}, \textit{BCL2}, and \textit{XIAP} were downregulated in bortezomib-treated human cells.

**Bortezomib Sensitivity Correlates with Basal NF-\kappa B Activity in KP Lung Adenocarcinoma Cells**

To investigate the dose-response profile of bortezomib, we treated a panel of KP and KP\textsuperscript{M} cells with increasing doses of bortezomib for 24 hours and monitored cell viability. As shown in Fig. 2A, KP and KP\textsuperscript{M} cell lines showed higher sensitivity to the drug than did control 3TZ and Kras-only LKR13 cells. We previously showed that in KP cell lines, NF-\kappa B p65 DNA-binding activity was consistently higher than in 3TZ and LKR13 cells (30). By measuring NF-\kappa B target gene expression, we observed that KP and KP\textsuperscript{M} cells also have higher NF-\kappa B target gene expression than do 3TZ or LKR13 cells (Supplementary Fig. S2). To examine whether the level of NF-\kappa B activity might be a biomarker to predict bortezomib response, we measured NF-\kappa B activity in KP cell lines, using ELISA (30), and quantified cell viability at 5 nM bortezomib treatment. In the cell lines assayed, NF-\kappa B activity was positively correlated with cytotoxicity (Fig. 2B), with cell lines that had high NF-\kappa B activity levels exhibiting more sensitivity to the drug than cells with lower NF-\kappa B levels. Thus, we conclude that lung adenocarcinoma cells with high NF-\kappa B signaling are dependent on continuous activation of this pathway. These
tumor volume at D4 compared with D0), whereas vehicle control–treated mice showed a 47.2% average increase in tumor volume. Thus, established tumors with Kras mutations and loss of p53 function are acutely sensitive to treatment with bortezomib.

To determine whether bortezomib affects tumors from the K model, which retains functional p53, we initiated lung tumorigenesis in \( \text{Kras}^{LSL-G12D/wt} \) mice with Adeno-Cre and treated the mice with a single 1 mg/kg dose of bortezomib at 20 weeks post infection (Supplementary Fig. S3B). As shown in Fig. 3C and D, K tumors did not regress upon treatment with bortezomib. Because tumors from KP mice had enhanced NF-\( \kappa \)B p65 nuclear localization when compared with K tumors (30), the different therapeutic response between KP and K tumors suggests that the effect of bortezomib may depend on the tumor genotype or the basal NF-\( \kappa \)B activity.

To compare the efficacy of bortezomib to that of other lung cancer therapies, we injected KP lung tumor cells s.c. into immunocompromised mice, allowed tumors to form, and then treated the mice with bortezomib. As shown in Supplementary Fig. S4, bortezomib markedly reduced tumor volumes in the short term and diminished tumor progression to a similar extent as cisplatin, a first-line chemotherapy for lung cancer (2).

data are consistent with studies showing that multiple myelomas with high NF-\( \kappa \)B activity are more sensitive to bortezomib (22) and IKK inhibitors (13).

**Bortezomib Leads to Lung Tumor Regression in KP Mice**

Our cell-based studies showed that bortezomib induced apoptosis in murine lung adenocarcinoma cell lines grown in culture (Fig. 1C). To understand the relevance of these findings in vivo, we examined bortezomib-mediated NF-\( \kappa \)B inhibition in both KP and K models of lung cancer. Previous data have shown that KP lung tumors have higher NF-\( \kappa \)B signaling than do those from the K model (30). To test the functional requirement for NF-\( \kappa \)B in KP tumors, we infected 6- to 8-week-old KP mice with adenoviruses expressing Cre (Adeno-Cre; Supplementary Fig. S3A). At 10 weeks after infection, mice were treated with a single dose of 1 mg/kg bortezomib, a maximum tolerated dose that has been shown to inhibit the proteasome and NF-\( \kappa \)B activity in mice (19, 35). Individual lung tumors were monitored using micro–computed tomography (microCT) imaging prior to treatment (D0) and 4 days post treatment (D4). As shown in Fig. 3A and B, a single dose of bortezomib resulted in significant tumor regression (55.4% average decrease in tumor volume at D4 compared with D0), whereas vehicle control–treated mice showed a 47.2% average increase in tumor volume. Thus, established tumors with Kras mutations and loss of p53 function are acutely sensitive to treatment with bortezomib.

To determine whether bortezomib affects tumors from the K model, which retains functional p53, we initiated lung tumorigenesis in \( \text{Kras}^{LSL-G12D/wt} \) mice with Adeno-Cre and treated the mice with a single 1 mg/kg dose of bortezomib at 20 weeks post infection (Supplementary Fig. S3B). As shown in Fig. 3C and D, K tumors did not regress upon treatment with bortezomib. Because tumors from KP mice had enhanced NF-\( \kappa \)B p65 nuclear localization when compared with K tumors (30), the different therapeutic response between KP and K tumors suggests that the effect of bortezomib may depend on the tumor genotype or the basal NF-\( \kappa \)B activity.

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Bortezomib Increases Survival in KP Mice

To analyze the long-term effects of bortezomib therapy, we investigated whether a 4-dose regimen of bortezomib (19) could prolong survival of tumor-bearing mice. Using a cohort of KP mice, we treated tumor-bearing animals 8 weeks after Adeno-Cre infection with bortezomib once a week for 4 weeks (Supplementary Fig. S3A). As shown in Fig. 5A, KP mice treated with 4 doses of bortezomib survived significantly longer (104.2 ± 19.8 days; P = 0.001) than did control-treated mice (76.8 ± 10.6 days; P = 0.36). This finding suggests that loss of p53, although a predictor of poor prognosis in some cancer therapies, still permits therapeutic benefits from bortezomib and indicates that this agent and possibly other NF-κB inhibitors may work selectively in tumors with high basal NF-κB activity.

Acquired Resistance Arises in KP Tumors after Bortezomib Treatment

Although the 4-dose regimen of bortezomib prolonged survival in the KP lung tumor model, the treated mice
eventually succumbed to their disease (Fig. 5A). To examine whether bortezomib-treated tumors relapse after repeated therapy, we administered bortezomib to another cohort of tumor-bearing KP mice once a week for 4 weeks. Tumor response was measured by twice-weekly microCT to determine the volume of individual lung tumors. As we had observed with short-term microCT imaging (Fig 3A), bortezomib treatment led to rapid KP tumor regression after the first dose (10.5 weeks; Fig. 5C) and delayed tumor growth, compared with vehicle control. However, by the 4th dose (13 weeks; Fig. 5C), tumors had become insensitive to treatment, suggesting that they had acquired resistance to the drug.

To investigate whether KP tumors present at the end of the treatment regimen were resistant to bortezomib-induced apoptosis, we treated KP mice, as described above, with 3 doses of bortezomib or vehicle control. After an additional week, mice were treated with a final dose of bortezomib and sacrificed 48 hours later. Tumors from mice that had received previous bortezomib treatments no longer demonstrated significant CC3 staining in response to a final dose of the drug (Fig. 5D, right), when compared with acutely treated naïve tumors (Fig. 5D, left). These data further suggest that the pretreated tumors had acquired resistance to bortezomib.

An Orthotopic Lung Tumor Model for Imaging Response and Resistance to Bortezomib

To facilitate imaging of the bortezomib treatment response, we adapted a transplantation-based orthotopic lung cancer mouse model (37). As outlined in Fig. 6A, we infected KP cell lines with a retrovirus expressing firefly luciferase. The cells were transplanted into immunocompetent recipient mice by i.v. injection, resulting in the development of in situ lung tumors that could be imaged and quantified by bioluminescence imaging.

To determine whether bortezomib therapy was effective in the orthotopic model, we transplanted 10,000 KP cells into host mice and treated the mice 5 weeks later, either with control or with a weekly 4-dose regimen of bortezomib (37). As in the autochthonous setting, in this model bortezomib treatment significantly increased survival, compared with control treatment (average survival: 47.0 ± 2.0 days for control and 64.0 ± 5.4 days for bortezomib; \( P = 1.4 \times 10^{-5} \); Fig. 6B). Bioluminescence imaging revealed a marked tumor regression at day 2 and day 4 after bortezomib treatment (Fig. 6C)—also reminiscent of the bortezomib-mediated lung tumor regression in the autochthonous model (Fig. 3A).

After the 2nd and 3rd bortezomib treatments, the lung tumor signal still decreased or stabilized. However, as with the tumor relapse detected in the autochthonous model, a 4th dose of bortezomib was ineffective (Fig. 6C), suggesting the relapsed tumors had become refractory to drug treatment.

To test whether the onset of bortezomib resistance was accompanied by increased NF-κB activity, we generated cell lines from orthotopic KP tumors that were treated either with 4 doses of bortezomib (resistant cell lines) or with vehicle controls (sensitive cell lines). As shown in Supplementary Fig. S5, resistant cells exhibited higher viability and increased colony formation, compared with sensitive cells, upon

Figure 5. Bortezomib increases survival in KP mice. A and B, Kaplan–Meier survival curve of vehicle control and bortezomib-treated KP (A) and K (B) mice. Arrows indicate weekly 1 mg/kg bortezomib regimen. C, quantification of lung tumor volume of control- and bortezomib-treated KP mice. From 10 weeks after Adeno-Cre infection, lungs were imaged by microCT to measure individual tumor volumes (n = 4). Data points represent means of fold change and SD relative to 10 weeks (set to 1). Arrows indicate bortezomib injection. D, CC3 staining (×200) in lung tumors receiving 3 doses of control (“C”) and 1 final dose of bortezomib (“B,” left) or 4 doses of bortezomib (right). Tumors were harvested 48 hours after the last treatment (n = 4).
Bay-117082 is a small-molecule compound that inhibits this IKK kinase activity (23). Like bortezomib, Bay-117082 has been shown to suppress NF-κB signaling in cells and mice (23, 24). Unlike bortezomib, which affects multiple cellular activities in addition to NF-κB, the use of Bay-117082 provides an extra degree of selectivity for inhibiting this pathway.

We found that Bay-117082 treatment induced caspase 3 cleavage and cell death in KP cell lines in vitro (Supplementary Fig. S10). We assessed the expression of prosurvival NF-κB target genes in these cells after Bay-117082 treatment and found that Bay-117082 downregulates NF-κB target genes, such as Bcl2, Bclxl, and Xiap (Fig. 7A). Next we tested Bay-117082 in our orthotopic lung tumor model (Fig. 7B). Using bioluminescence imaging, we observed that Bay-117082 treatment significantly reduced lung tumor signal in the initial phase (Fig. 7B; 0–9 days) and delayed lung tumor progression until 42 days (Fig. 7B). However, treated tumors eventually became refractory to therapy and progressed in the lung and distant organs despite continuous Bay-117082 treatment (see 42–63 days in Fig. 7B). These data suggest that the relapsed tumors acquired resistance to the drug.

Finally, to gain insight into the survival benefit of Bay-117082, we treated a cohort of KP mice (8 weeks post Adeno-Cre) with Bay-117082 (10 mg/kg), 3 times per week by i.p. treatment (Supplementary Fig. S5A and B). Of importance, when treated with bortezomib in culture, resistant cells showed robust downregulation of NF-κB target genes regulating apoptosis and survival (Supplementary Fig. S6), indicating bortezomib was still effective in inhibiting the NF-κB pathway. Sensitive and resistant cell lines were next used to evaluate the activity of both the canonical and the noncanonical NF-κB pathways. Immunoblots, ELISA, and immunofluorescence analysis showed similar levels of cytoplasmic and nuclear NF-κB subunits in the resistant and sensitive cells (Supplementary Figs. S7 and S8). Moreover, transcriptional profiling of 10 NF-κB target genes did not reveal a globally increased expression of NF-κB targets in resistant tumor cells (Supplementary Fig. S9). These data suggest that cells generated from bortezomib-resistant lung tumors harbor levels of basal NF-κB activity similar to those in sensitive cells.

The NF-κB Inhibitor Bay-117082 Shows Therapeutic Efficacy In Vivo

To expand the scope of pharmacologic inhibition of NF-κB, we next tested a compound that was developed as an inhibitor of IKK. IKK-mediated IkB phosphorylation is required for IkB degradation and NF-κB activation (8), and Bay-117082 is a small-molecule compound that inhibits this IKK kinase activity (23). Like bortezomib, Bay-117082 has been shown to suppress NF-κB signaling in cells and mice (23, 24). Unlike bortezomib, which affects multiple cellular activities in addition to NF-κB, the use of Bay-117082 provides an extra degree of selectivity for inhibiting this pathway.

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injection for 4 weeks, a dose previously shown to inhibit NF-κB in mice (24). As seen in Fig. 7C, the KP mice treated with Bay-117082 survived significantly longer than mice treated with vehicle control (79.0 ± 13.2 days for control and 120.2 ± 27.0 days for Bay-117082; P = 0.008). In a short-term treatment, BAY-117082 led to apoptosis, as indicated by cleavage of caspase 3 (Supplementary Fig. S11). Altogether, these results indicate that IKK inhibition has therapeutic efficacy in lung cancer.

**DISCUSSION**

As already noted, the NF-κB pathway has recently shown great promise as a cancer therapeutic target (7). Large-scale RNA interference screens and mouse model studies have documented that components of the NF-κB signaling pathway are required for the survival of lung cancer cells and other cancer cell types (30, 38). Our study evaluated the efficacy of pharmacologically targeting NF-κB in lung cancer. Our data showed that bortezomib, used as a single agent, provided significant survival advantage in a KRasG12D-driven p53-deficient lung cancer model. Bay-117082, an IKK inhibitory compound, also provided a survival advantage, although at a more frequent dosing schedule than that of the bortezomib regimen. Our study provides evidence that the NF-κB pathway is a potential therapeutic target in lung cancer, and with further characterization of NF-κB genetic mutations and NF-κB target gene expression profiling in these types of tumors, NF-κB inhibitors may become an important option for lung cancer targeted therapy.
therapies. Our autochthonous model not only recapitulates important genetic and pathologic features of human lung adenocarcinoma but also provides a physiological tumor microenvironment to study therapeutic response. The orthotopic model, a variant of this approach, is based on the isolation of mouse lung adenocarcinoma cells from genetically tractable primary mouse lung tumors, followed by their seeding into the lungs of immunocompetent recipient mice (37). This latter model is also important for its ability to accelerate cancer treatment studies in mice, by allowing rapid imaging of therapeutic response and ex vivo genetic modification of cells by introduction of short hairpin RNAs or cDNAs. Using both systems, we have shown that mouse models, when combined with in vivo imaging and tumor biomarker analysis, can serve as a powerful platform to identify and validate novel cancer therapies. These mouse models will provide valuable preclinical information to be cross-compared with clinical trial data in human patients. Such studies could potentially dissect molecular mechanisms of selected drugs and identify biomarkers to predict patient response.

We have shown that bortezomib treatment induced apoptosis in lung tumors driven by activated Kras and lacking p53. Apoptosis may be one of the mechanisms underlying the significant decrease in lung tumor burden achieved by this drug in the KP model. We performed molecular characterization in cultured KP cells to show that bortezomib reduced expression of antiapoptotic NF-κB target genes (eg, Bcl2, Bclxl, Birc2, and Xap1; Fig. 1B). This result is consistent with a prosurvival function of NF-κB in normal and cancer cells (8). Previous studies have developed inhibitors for Bcl2 family proteins (ABT-737; ref. 39) and cIAP1 (40, 41) as novel cancer therapies, but considering the simultaneous suppression of many antiapoptotic genes observed in bortezomib-treated cells (Fig. 1B), NF-κB inhibition appears to provide a promising approach to lower the apoptosis threshold in cancer cells. Because human tumors often upregulate NF-κB signaling to gain resistance to chemotherapy (12), NF-κB inhibitors may also serve as chemosensitizing agents in combination therapies.

Of note, bortezomib and Bay-117082 have differential effects in the transcriptional profile of certain NF-κB targets in vitro. For example, Bcl2 and Myc inhibition was more robust upon bortezomib treatment, whereas Xap1 inhibition was stronger upon Bay-117082 treatment (Fig. 1B and Fig. 7A). Although Bay-117082–treated mice survived slightly longer than the bortezomib-treated cohort, these 2 groups were not statistically significant (P = 0.103), and this effect may be due to a more frequent dosing schedule with Bay-117082 (3 injections per week) than with bortezomib. Our treatment data suggested that the efficacy of bortezomib is dependent on the genetic context of lung tumors. Our previous study showed that genetic inhibition of NF-κB by a IκB super-repressor (a dominant negative form of IκB) or knockdown of p65/RelA or NEMO preferentially triggered cell death in KP cells, but not in 3TZ or LKR13 cells (30). In this treatment study, KP cells also showed greater bortezomib sensitivity than did 3TZ or Kras-only cells. In vivo, KP tumors with high NF-κB activity were sensitive to bortezomib, whereas Kras-only tumors with lower activity were not responsive, which is consistent with clinical data showing that an NF-κB signature in multiple myeloma patients is associated with a better treatment outcome with bortezomib (22). Phase II clinical trials indicated that bortezomib has modest effects in advanced NSCLC patients previously treated with chemotherapy (42). Our observations that bortezomib sensitivity correlates with NF-κB activity suggest that NF-κB is a major target of this drug and NF-κB pathway activity may serve as a biomarker to predict the therapeutic response of bortezomib or other NF-κB inhibitory drugs.

In addition to inhibiting NF-κB, bortezomib and Bay-117082 have known multitargeted effects. Bortezomib can also stabilize the CDK inhibitors p21 and p27 (43), whereas Bay-117082 can stimulate the stress-activated protein kinases, p38 and JNK-1 (23). New classes of more selective NF-κB inhibitors, such as ATP analog IKK inhibitors, will improve efforts to drug the NF-κB pathway in cancer. Moreover, the therapeutic inhibition of NF-κB has thus far been viewed with caution owing to this pathway’s diverse functions in different physiological contexts such as the immune system. Despite these cautions, bortezomib has been extensively used in the clinic with manageable side effects. Future work will be required to address the safety profile of Bay-117082 or related molecules and their impact on noncancerous tissue.

We further observed that prolonged bortezomib treatment led to resistance in KP lung tumors. Acquired bortezomib resistance has been reported in the literature (44), and our results are in agreement with clinical findings that multiple myelomas initially responsive to bortezomib often relapse and become resistant to the drug (44). Several studies have suggested possible mechanisms of bortezomib resistance, such as (1) mutations or overexpression of the PSMB5 subunit of 26S proteasome (45), (2) overexpression of HSP27 (46), and (3) increased activity of the aggresome pathway (44). Of interest, basal NF-κB activity is not increased in bortezomib-resistant lung tumor cell lines, at least in vitro (Supplementary Figs. S5–S9). Our studies establish a physiologically relevant system to explore the mechanisms of bortezomib resistance in lung cancer.

In summary, we have characterized therapeutic response and resistance to NF-κB inhibitors in several mouse models of lung cancer. In vivo treatment with bortezomib or Bay-117082 significantly reduced tumor volume and increased survival in mice with lung tumors associated with high NF-κB activity. However, repeated treatment resulted in the emergence of drug-resistant tumors, which may recapitulate important features that will occur in human patients. Mouse models will undoubtedly be useful for studying additional NF-κB inhibitors, as well as combination therapies.

**METHODS**

**Mice and Drug Treatment**

The Massachusetts Institute of Technology (Cambridge, MA) Institutional Animal Care and Use Committee approved all animal studies and procedures. To initiate lung tumors, cohorts of K or KP mice of 129sv/jae background were infected with 2.5 × 10^7 plaque-forming units of Adeno-Cre (University of Iowa) by intranasal...
inhalation, as described previously (2, 28). Mice were given bortezomib (LC Labs) in PBS [0.5% dimethylsulfoxide (DMSO)] at 1 mg/kg body weight i.v., as indicated. Bay-117082 (CalBiochem) was dissolved in DMSO, diluted in PBS as a fine suspension, and injected at 10 mg/kg body weight i.p., as indicated.

**Immunohistochemistry**

Mice were sacrificed by carbon dioxide asphyxiation. Lungs were inflated with 4% formalin (neutral buffered formalin), fixed overnight, and transferred to 70% ethanol. Lung lobes were embedded in paraffin and sectioned at 4 μm and stained with H&E for routine pathologic study. For staining with anti-CC3 antibodies (#9661; Cell Signaling), lung tumor sections were dewaxed, rehydrated, and subjected to high-temperature antigen retrieval—10 minutes of boiling in a pressure cooker in 0.01 M citrate buffer, pH 6.0. Slides were stained overnight at 4° in 1:100 primary antibody. A goat antirabbit horseradish peroxidase-conjugated secondary antibody (Vector Laboratories) was used at 1:200 dilution, incubated for 1 hour at room temperature, followed by diaminobenzidine staining (Vector Laboratories). The number of positive cells per tumor area was quantified using Biocuant software from >10 tumors in ≥3 mice per group (2).

**MicroCT and Bioluminescence Imaging**

At indicated time points, mice were scanned for 15 min under isoflurane anesthesia, using a small animal xExplore Locus microCT (GE Healthcare) at 45-μm resolution, 80 kV, with 450-mA current (47). Images were acquired and processed using GE eXplore software. Bioluminescence imaging was performed as previously described (48). Mice were imaged for 60 seconds, and signals in the lung were quantified using Xenogen software.

**Immunoblotting and Immunofluorescence**

Cell pellets were lysed in Laemmli buffer. Equal amounts of protein (16 μg) were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Blots were probed with antibodies (1:1000 dilution) against p65 (c-20), NEMO (FL-419), Parp (46D11), c-Rel (C), p100/p52 (#4882; Cell Signaling), or CC3 (#9661; Cell Signaling). Nuclear-cytoplasmic fractionations and NF-κB p65 DNA-binding activity assay were as described recently (30). For the NF-κB subunit DNA-binding activity assay, 5 μg of nuclear extracts was used to determine NF-κB DNA-binding activity in an ELISA-based assay, according to the manufacturer’s instructions (TransAM; Active Motif). Immunofluorescence was performed as recently described (49). Antibodies are as follows: p65 (c-20, 1:100), p100/p52 (#4882, 1:100; Cell Signaling), and goat antirabbit Alexa 488 (1:1000; Invitrogen). Cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and mounted in Vectashield antifade mountant (Vector Laboratories). The number of positive cells per tumor area was quantified using Biocuant software from >10 tumors in ≥3 mice per group (2).

**Cell Viability Assay**

Cell culture conditions were as described recently (30). Cells were split into 96-well plates (5,000 cells per well). After 24 hours, cells were treated with bortezomib, and 24 hours later cell viability was measured by the Cell Titer Aqueous Kit (Promega) in triplicates. Vehicle control–treated cell values were set to 1 (100% viability). For Fig. 2A and Supplementary Fig. S5A, the data are representative of 2 independent experiments.

**Gene Expression Analysis**

RNA was purified using TRIzol (Invitrogen), according to the manufacturer’s instructions. One microgram of RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR (quantitative PCR) amplification was performed using Taqman probes (Applied Biosystems). Data were normalized to the Gapdh (mouse) or GAPDH (human) levels.

**Statistics**

P values were determined by Student t tests.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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